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Use of Single-Chain Antibody Derivatives for Targeted Drug Delivery

Yaghoub Safdari,[1,7] Vahideh Ahmadzadeh,[2,3] Masoumeh Khalili,[4] Hossein Zarei Jaliani,[5] Vahid Zarei,[6] and Vahid Erfani-Moghadam[1,7]

[1]Medical Cellular and Molecular Research Center, Golestan University of Medical Sciences, Gorgan, Iran; [2]Drug Applied Research Center, [3]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [4]Golestan Research Center of Gastroenterology and Hepatology, Golestan University of Medical Sciences, Gorgan, Iran; [5]Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran; [6]Department of Chemistry, College of Sciences, Shiraz University, Shiraz, Iran; and [7]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Golestan University of Medical Sciences, Gorgan, Iran

Single-chain antibodies (scFvs), which contain only the variable domains of full-length antibodies, are relatively small molecules that can be used for selective drug delivery. In this review, we discuss how scFvs help improve the specificity and efficiency of drugs. Small interfering RNA (siRNA) delivery using scFv-drug fusion peptides, siRNA delivery using scFv-conjugated nanoparticles, targeted delivery using scFv-viral peptide-fusion proteins, use of scFv in fusion with cell-penetrating peptides for effective targeted drug delivery, scFv-mediated targeted delivery of inorganic nanoparticles, scFv-mediated increase of tumor killing activity of granulocytes, use of scFv for tumor imaging, site-directed conjugation of scFv molecules to drug carrier systems, use of scFv to relieve pain and use of scFv for increasing drug loading efficiency are among the topics that are discussed here.
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INTRODUCTION

Targeted drug delivery systems have several advantages over traditional systems, including more safe movement through the cell membrane, reduced side effects and a higher efficiency (1–3). Antibody molecules, which specifically react with particular antigens on the cell surface, can serve as appropriate targeting elements/carriers for targeted drug delivery. Full-length antibodies are extensively used as the targeting elements of drug delivery systems (4–7). The major drawback correlated with this type of antibodies is their large size, which may negatively affect their potency. Constant domains of antibodies are not directly involved in antigen recognition and binding. Therefore, single-chain antibodies (scFvs), which are devoid of constant domains, are still able to bind their specific antigens (8,9). Because of their small size, scFvs are expected to display a higher cell penetration rate than their full-length counterparts; therefore, they are more favorable for use as targeting elements in drug delivery systems (10). This review includes the most updated studies regarding the use of scFvs as targeting elements of drug delivery systems and presents innovative strategies for increasing the efficiency of drug delivery systems, including liposomal and nonliposomal drug carriers.

Address correspondence to *Vahideh Ahmadzadeh, Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. P.O. Box: 5165665813, Fax: +98 4133323234; E-mail: Vahideh_Ahmadzadeh@yahoo.com; and Masoumeh Khalili, Golestan Research Center of Gastroenterology and Hepatology (GRCGH), Golestan University of Medical Sciences, Gorgan, Iran. P.O. Box: 49341746110T; E-mail: mkhalili_ps@yahoo.com.*
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USE OF SCFV MOLECULES FOR SIRNA DELIVERY

Efficient Delivery of siRNA to Breast Cancer Cells Using $(Arginine)_9$–Anti-HER2-scFv Fusion Peptide

Small interfering RNAs (siRNAs), which interfere with expression of mRNA molecules, have gained enormous attention for cancer treatment (11,12). A major drawback correlated with nontargeted siRNA delivery systems is the lack of specificity for the target cells. Jiang and colleagues used an anti-HER2-scFv antibody to specifically deliver siRNA molecules to HER2-overexpressing breast

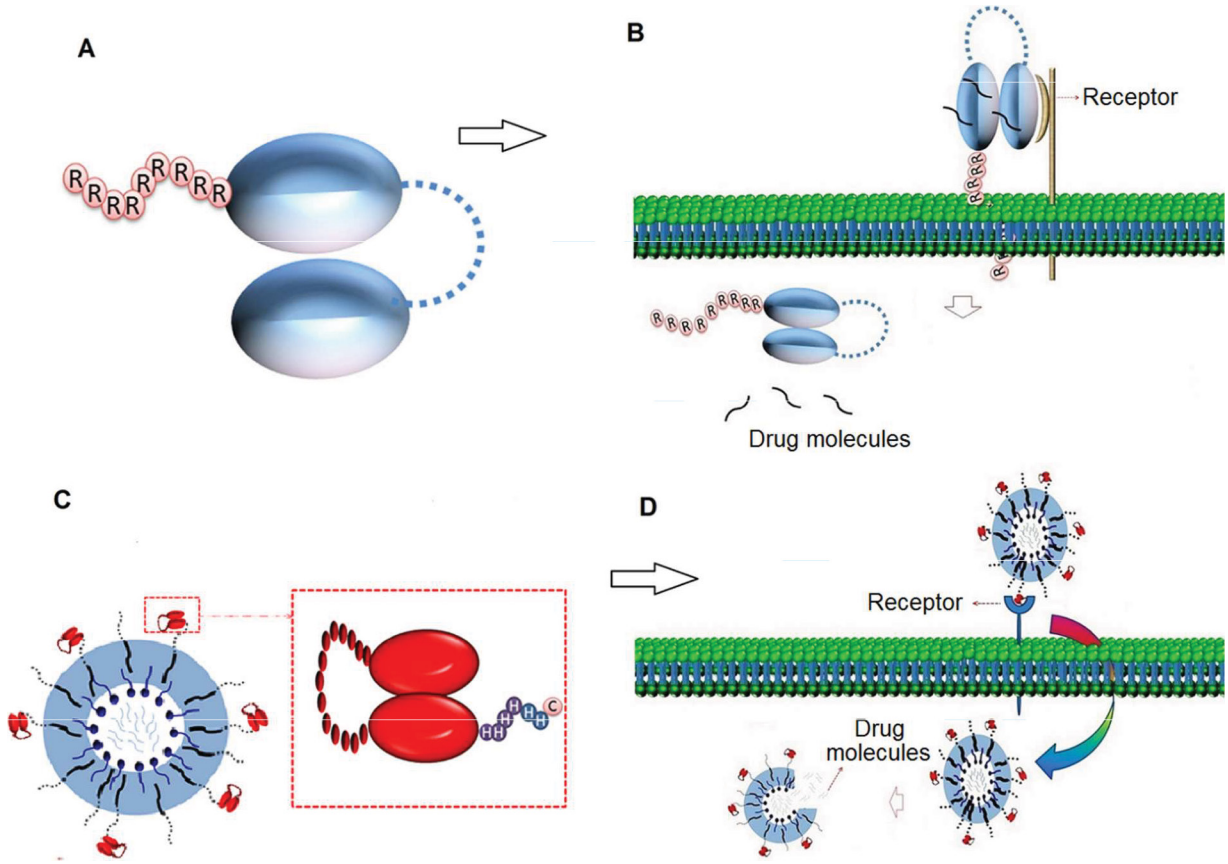

Figure 1. Structure and action mechanism of scFv-arginine fusion peptide and scFv-based immunoliposome (IL). (A) Nine arginine residues act as a cell-penetrating peptide, which help the fusion peptide enter the cell. (B) Fusion peptide binds to a specific receptor on the target cell by its scFv moiety and enters the cell by its C-terminal tail arginine resides. (C) Cysteine residue at the C-terminal end allows the scFv to form a covalent bond with PEG–PLA nanoparticles to create IL. (D) IL binds to the target cell through its scFv, becomes internalized into the cell and releases the encapsulated drugs.

cancer cells to suppress the expression of chemokine receptor 4 (CXCR4), a protein that plays a main role in cell survival and cancer metastasis. The anti-HER2-scFv contained nine arginine residues at its C-terminal end (Figure 1A) (1). Arginine residues are widely used to deliver nucleic acids into cells; they act as a cell penetration peptide that binds the cells and transduces the siRNA molecules across the plasma membrane (Figure 1B) (13). The anti-HER2-scFv significantly increased the delivery of the siRNA molecules to HER2-positive BT-474 breast cancer cells and tumor xenografts, while it was unable to deliver siRNA molecules to MDA-MB-231 cells and tumor xenografts. MDA-MB-231 cells do not express HER2 receptors on their surface, and as a result, anti-HER2-scFv molecules cannot recognize them. Reduced tumor metastasis and prolonged animal survival have been the results of anti-HER2-scFv-mediated delivery of anti CXCR4-siRNAs to BT-474 xenograft-bearing mice (1).

siRNA Delivery Using $(Arginine)_9$-Anti-EGFR-scFv Fusion Peptide to Overcome Drug Resistance in Lung Cancer Cells

Epidermal growth factor receptor (EGFR) overexpression occurs in many types of human cancers including lung cancer (14–16). Tyrosine kinase inhibitors (TKIs), which inhibit the tyrosine kinase activity of the EGFR intracellular domain, can suppress cancer progression. However, after a variable period of time, patients acquire resistance to such inhibitors for several reasons, including point mutations in the EGFR intracellular tyrosine kinase domain which annuls the binding of TKI to EGFR, mutation and subsequent activation of Kirsten rat sarcoma viral oncogene (KRAS), epithelial-to-mesenchymal transformation, gene amplification of mesenchymal-to-epithelial transformation (MET) factor and so forth (17–20). To overcome TKI resistance in lung cancer, Lu and colleagues produced a single-chain format of nimotuzumab, an anti-EGFR monoclonal antibody, and used it to specifically deliver siRNA molecules to EGFR-expressing cells to suppress *MET*, *EGFR* and *KRAS* gene expression (21). The scFv contained

nine additional arginine residues (9R) at its C-terminal end that guaranteed the penetration of fusion peptide (scFv-9R), and therefore delivery of siRNAs, into EGFR-positive cells. Control scFvs, which were devoid of C-terminal 9R, were unable to deliver siRNA molecules. siRNA molecules were loaded on scFv molecules by simply mixing, and not by covalent bonds. EGFR-negative cancer cells (H69 cells) did not internalize siRNA-loaded fusion peptide, indicating that scFv moiety played a pivotal role in specificity of drug delivery to EGFR-expressing cells. scFv-9R-mediated siRNA delivery to H1993, H1975 and A549 cancer cells decreased the expression of *MET*, *EGFR* and *KRAS* genes, respectively. These cell lines carry MET amplification, L858R/T790M EGFR mutation and KRAS mutation, respectively. Delivered siRNA molecules were able to suppress the growth of these cell lines significantly and restored their sensitivity to gefitinib (a TKI). Capacity of scFv-9R for siRNA delivery has been further confirmed in an animal model in which EGFR-specific siRNA decreased the growth of H1975 xenografts in nude mice and prolonged the survival time (21).

Covalent Conjugation of Anti-HER2-scFv to PEG–PLA Nanoparticles for siRNA Delivery to Cancer Cells

Polyethylene glycol–poly(D,L-lactide) (PEG–PLA)-based nanoparticles have been extensively used to encapsulate and deliver siRNA molecules to cancer cells. Dou and colleagues used this type of nanoparticles for delivery of antipolo-like kinase 1 (Plk1, a kinase enzyme involved in cell division) siRNA to breast cancer cells (22). They conjugated an anti-HER2-scFv antibody to the surface of siRNA-loaded PEG–PLA nanoparticles and evaluated the anticancer activity of the resultant conjugate on breast cancer cells. The scFv contained an additional cysteine residue at its C-terminal end (outside of antigen-binding sites, after 6-amino-acid His-Tag, Figure 1C) which allowed it to covalently bind to PEG–PLA nanoparticles via a thiol-maleimide coupling reaction (22). The thiol-maleimide reaction is a process that is widely used to bioconjugate biomolecules, including proteins (23,24). Site-directed conjugation permits the scFv to conjugate with another molecule without losing affinity. Loss of affinity in nondirected conjugation may be due to induction of conformational change or blockage of antigen-binding sites (25). The anti-HER2-scFv-coated PEG–PLA nanoparticles ($scFv_{HER2}$–NP_{siRNA}) were able to selectively deliver anti-Plk1 siRNA to BT474 cells (HER2-overexpressing breast cancer cells) but not to HER2-negative MDA-MB-231. Compared with nontargeted nanoparticles, the scFv-loaded nanoparticles ($scFv_{HER2}$–NP_{siRNA}) were more potent in silencing of *Plk1* gene in BT474. Nontargeted nanoparticles were able to suppress the growth of both BT474 and MDA-MB-231 cells, indicating that they enter the cell in an unspecific manner. In contrast, $scFv_{HER2}$–NP_{siRNA} caused significant growth inhibition only in the BT474 cells, demonstrating that the scFv portion increases the specificity to target cells. Using fluorescence imaging, Dou and colleagues demonstrated that scFv molecules present on the surface of the nanoparticles (Figure 1C, D) significantly increased the tumor penetration rate (BT474 tumor xenografts) (22). These results all indicate that scFv molecules offer specificity to target cells and further increase the efficacy of PEG–PLA nanoparticles in drug delivery.

Targeted siRNA Delivery to Cancer Cells Using a Fusion Peptide Composed of Truncated Protamine and a Cetuximab-Derived scFv

Anti-EGFR scFv antibody has also been used for drug delivery to cervical cancer cells. Zhang and colleagues produced a fusion peptide composed of variable domains of cetuximab (joined together with a short flexible linker, GGSSR SSSSG GGGSG GGG) and a truncated form of protamine (t-protamine, containing 22 amino acids) and used it for siRNA delivery to HeLa cells to suppress human wings apart-like (*hWAPL*) gene expression (26). Expression of this gene has been discovered to increase in human papilloma virus–related cervical cancer. t-Protamine has the ability to bind DNA; therefore, scFv-protamine fusion peptide is expected to retain DNA-binding activity. Using enzyme-linked immunosorbent assay (ELISA) and gel shift assay, Zhang and colleagues showed that the fusion peptide retained both EGFR and DNA-binding activities. Expression of hWAPL mRNA was strikingly reduced in HeLa cells upon fusion peptide–mediated intranucleus delivery of hWAPL siRNA. Cell proliferation rate was also significantly reduced (26). These results indicate that the scFv-t-protamine fusion peptide effectively delivered the siRNA molecules to the nucleus of target cancer cells.

siRNA-Mediated Gene Silencing in Dendritic Cells Using scFv-Coated Lipid Nanoparticles

Dendritic cells (DCs) play a critical role in immune responses (27), and therefore manipulation of their gene expression profile will influence their immunomodulatory capacities. Inhibition of immune responses can help treat autoimmune diseases. DEC205 is a receptor expressed by DCs. Katakowski and colleague took advantage of this receptor for siRNA delivery to DCs to suppress immune responses (28). They produced a single-chain format of an anti-DEC205 monoclonal antibody and conjugated it to the surface of siRNA-loaded lipid nanoparticles. The scFv contained at its C-terminal end a 10-residue His-tag, a short glycine-serine linker (G4S) and an additional cysteine residue, allowing the site-directed conjugation to lipid components of siRNA delivery system (Figure 2A, B). scFv molecules bind lipids through maleimide groups. siRNA molecules had been designed to target *CD40*, *CD80* and *CD86* genes. To examine whether the resultant immunoliposomes (ILs) are specific to DEC205, Katakowski and colleagues treated DEC205-positive

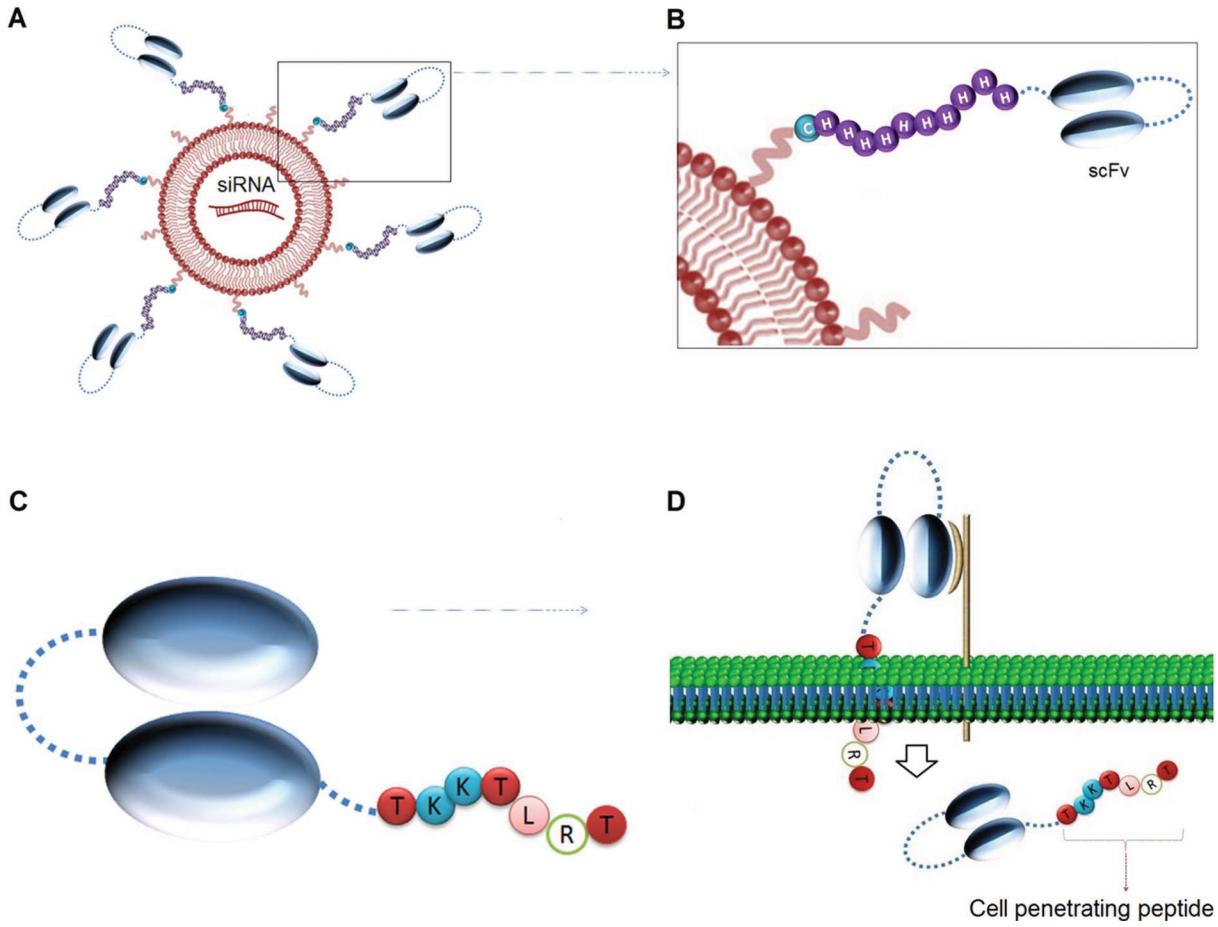

Figure 2. Schematic view of a siRNA-loaded immunoliposome (IL) and a short peptide–scFv fusion peptide. (A) scFv molecules bind to PEG particles on the surface of siRNA-loaded IL to form a targeted siRNA carrier. (B) Targeting ligand of the IL consists of a scFv molecule, a short linker, 10 histidine residues and a cysteine residue that involves in covalently binding to PEG particles. (C) Amino acid sequence of TKKTLRT forms a short peptide with cell penetration capacity. (D) This short peptide allows the fusion peptide to enter the target cells previously recognized by scFv moiety.

and DEC205-negative CHO cells with ILs carrying Dy547-labeled siRNA (28). ILs showed approximately four-fold greater binding to DEC205-positive cells compared with nontargeted liposomes. Intravenous injection of fluorescently labeled ILs in B6 mice confirmed the role of anti-DEC205 scFv in specificity of the carrier; significant uptake of lipid nanoparticles was observed only in DEC205-positive DCs. The siRNA-loaded ILs significantly reduced the expression of *CD40*, *CD80* and *CD86* genes in DCs (28). These results all indicate that anti-DEC205 scFv serves as an efficient targeting element on the surface of liposomes and plays a critical role in the specificity of drug carrier systems.

scFv-Mediated siRNA Delivery to Avian Influenza Virus–Infected Cells

siRNA molecules have been found to be useful in controlling several viruses, including hepatitis B virus, dengue virus, adenovirus, respiratory syndrome virus, etc. (29–32). scFv molecules can be engaged to increase the specificity and efficiency of siRNA delivery to virus-infected cells. Infected cells usually express viral antigens on their surface that can serve as a target for antibody. Khantasup and colleagues exploited one of such antigens, hemagglutinin (HA), to specifically deliver siRNA molecules to highly pathogenic avian influenza virus (H5N1)-infected cells (33). The siRNA had been designed to inhibit the expression of the virus nucleoprotein mRNA. They employed a humanized anti-HA scFv on the surface of liposome-encapsulated siRNA molecules to form ILs that were able to specifically target H5N1-infected cells, which express high level of viral HA on their surface. The nucleotide sequence of the anti-H5N1 nucleoprotein was 5′-GGAUC UUAUU UCUUC GGAGd TdT-3′ for the sense strand and 5′-CUCCG AAGAA AUAAG AUCCd TdT-3′ for the antisense strand. Using immunofluorescence assay, Khantasup and colleagues demonstrated that ILs bound to HA-expressing cells (baculovirus-infected Sf9 cells) much more

efficiently than nontargeted liposomes (33). They also compared the antiviral activity of both ILs and nontargeted liposomes on H5N1-infected Madine-Darby canine kidney cells to discover how much the scFv improves the siRNA delivery system. ILs were found to be more potent than nontargeted liposomes in reducing viral titer. These results indicate that the scFv plays a pivotal role in internalization of siRNA into virus-infected cells (33).

TARGETED SIRNA DELIVERY TO MACROPHAGES AND COLONIC CELLS TO REDUCE COLITIS

CD98 is a cell surface receptor found to be overexpressed in colonic tissues of mice with colitis, on the surface of intestinal T cells ($CD4^+$ T cells and $CD8^+$ T cells) and B cells of patients with inflammatory bowel disease (IBD) and in intestinal macrophages (34–36). As CD98 plays an important role in progression of IBD, it can serve as a potential target for therapy of IBD patients. Xiao and colleagues took advantage of this cell surface antigen to specifically target colonic epithelial cells and macrophages to reduce colitis (37). They applied an anti-CD98 scFv on the surface of anti-CD98 siRNA-loaded nanoparticles. The nanoparticles were composed of PEG, urocanic acid and low-molecular-weight (2 kDa) polyethylenimine (PEI) (37). It has been demonstrated that the molecular weight of PEI plays an important role in terms of cytotoxicity; the lower the molecular weight, the higher the cell viability and transfection efficiency (38). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay revealed that scFv-loaded nanoparticles did not alter the viability of Colon-26 cells and RAW 264.7 macrophages, while PEI/siRNA complex induced significant cytoxicity. The scFv-loaded nanoparticles were able to enter Colon-26 cells, RAW 264.7 macrophages, bone marrow–derived macrophages and colitis tissues and exhibited excellent lysosomal escape ability. In Colon-26 cells and RAW 264.7 macrophages, the scFv-loaded nanoparticles reduced the level of CD98 significantly. In the mice with colitis, oral administration of hydrogel-encapsulated scFv-loaded nanoparticles reduced the colon expression of CD98 and diminished the severity of colitis (37). These results all indicate that scFv plays a pivotal role in the specificity of the siRNA delivery system and can serve as a suitable targeting ligand on the surface of drug carriers to target CD98-expressing cells.

USE OF SCFV FOR SMALL MOLECULE DELIVERY

Delivery of the Collagen-Binding Domain to Collagen-Enrich Carcinoma Using Anti-EGFR scFv

As a main constitute of extracellular matrix (ECM), collagen can potentially act as a barrier to drug absorption; therefore, it is considered an important target for cancer therapy. A short peptide sequence (TKKTLRT), well known as collagen-binding peptide (CBP), has been proven to specifically target collagen type 1 and significantly increase drug penetration. Liang and colleagues produced a fusion peptide composed of CBP and a cetuximab-based scFv antibody (expressed in *Pichia pastoris*) (Figure 2C, D) and evaluated its activity on A-431 cells and xenografts (10). A-431 is an EGFR-overexpressing skin cancer cell line. They found using immunocytochemistry and flow cytometry analysis that the cetuximab-based scFv bound to EGFR with the same affinity as that of full-length cetuximab. The fusion peptide was able to induce apoptosis and inhibit cell proliferation *in vitro*. They also found that CBD–scFv fusion protein penetrated the tumors more rapidly than intact cetuximab and significantly reduced the tumor growth, demonstrating that scFvs are more amenable than their full-length counterparts for use as targeting ligands of drug carriers. Besides binding to collagen, CBD has been proven to enhance the retention time of scFv in tumors (10).

Safe and Targeted Drug Delivery Using scFv-Viral Peptide Fusion Protein

Antibody-drug conjugates can be internalized from the cell surface through receptor-mediated endocytosis. Inside the endosomes, drug molecules may become inactive due to the acidic environment; therefore, they must escape from endosome to cytoplasm before lysosomal degradation (39,40). Use of Sendai virus F-protein has been proposed to avoid endosomal degradation. For safe and effective delivery of doxorubicin to cancer cells, Kumar and colleagues used a virus-based carrier, a fusion peptide composed a scFv [anti-PAP (placental isozyme of alkaline phosphatase)-scFv] and a part of Sendai virus F-protein (transmembrane domain and a part of cytoplasmic domain) (2). F-protein of the virus plays an important role in membrane fusion and delivery of viral genetic materials to the host cells (41). The fusion protein (immuno-virosome) was able to selectively recognize, and deliver doxorubicin molecules to, PAP-expressing cells [PAP-transfected HeLa and SaOs(T) cell lines] but not to fibroblast activation protein (FAP)-negative cells [CHO and SaOs(UT) cells]. The immuno-virosomes were found to deliver doxorubicin molecules to the cytoplasm of FAP-expressing cells chiefly through membrane fusion and not by endocytosis (2). Treatment of HeLa cells with cytochalasin B, which inhibits endocytosis, resulted in only a slight decrease in immuno-virosome internalization, indicating that endocytosis has not been the main way of internalization. These results indicate that the carrier (immuno-virosome) recognizes the target cells by its scFv moiety and enters these cells by its viral section.

Increased Specificity, Internalization Rate and Cytotoxicity Using scFv Diabody-Mediated Drug Delivery

Single-chain antibodies are usually produced by linking VH and VL domains of antibodies via a flexible peptide linker (composed of ~15 amino acids) that allows the assembly of domains to form paratopes, the antigen-binding sites (9). By shortening the linker between two adjacent VL and VH domains, they fail to assemble to form a regular scFv; however, they can assemble with

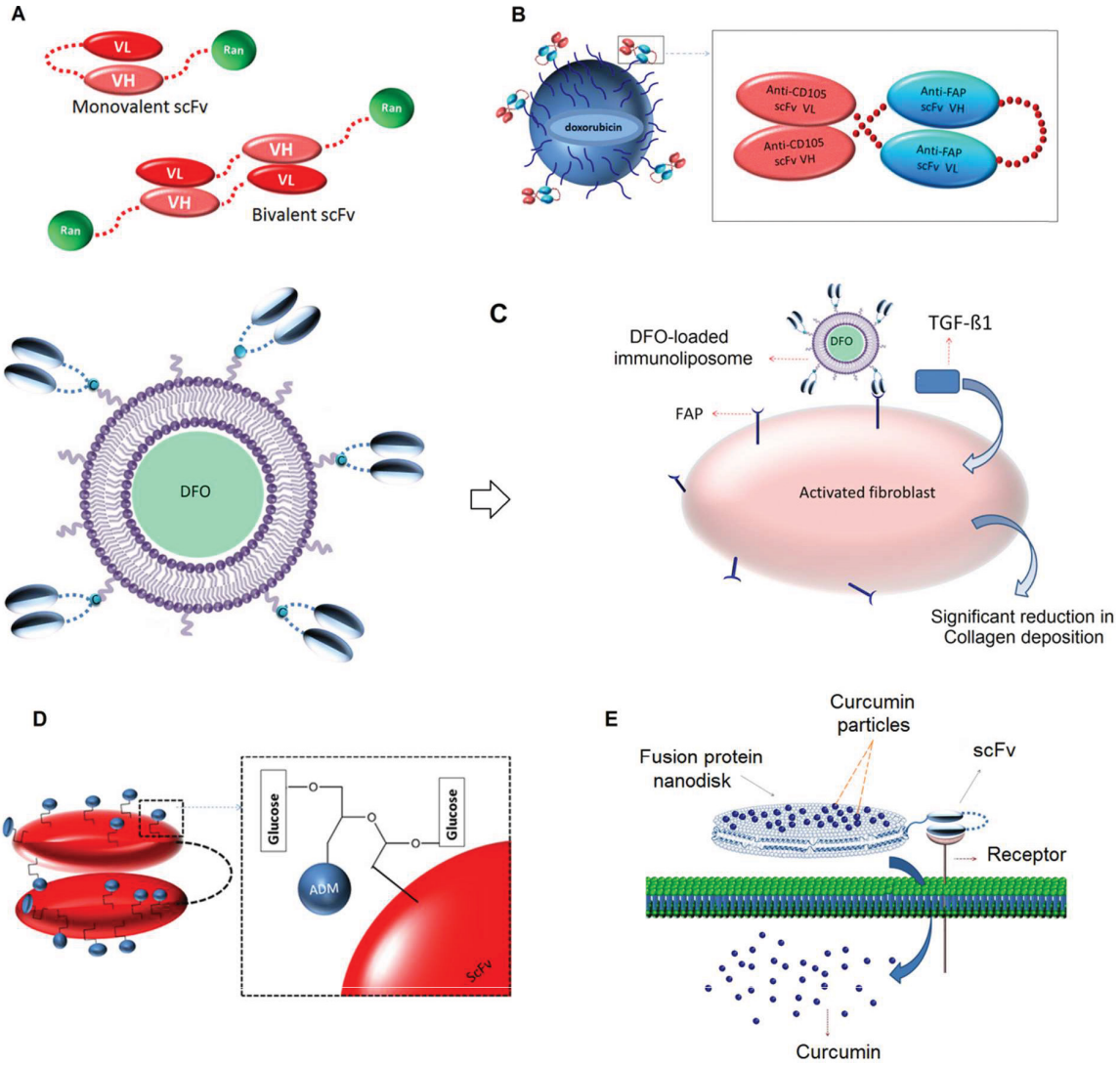

Figure 3. Structure and/or functional mechanism of some scFv-based drug carriers. (A) Monovalent and bivalent formats of Ranpirnase–scFv fusion peptide. Ranpirnase and scFv are joined together with a linker peptide. (B) Schematic view of a doxorubicin-loaded bi-specific IL. The targeting ligand is a fusion peptide composed two scFv molecules, anti-FAP scFv and anti-CD105 scFv. (C) Deferoxamine (DFO)-loaded anti-FAP IL. Anti-FAP scFvs contain a cysteine residue in their linker region that makes a covalent bond with PEG molecules on the surface of liposomes. The resultant ILs are able to bind FAP proteins expressed on the surface of TGF β1-activated fibroblasts and reduce collagen deposition. (D) Adriamycin (ADM)-loaded scFv molecule. Dextran can serve as a linker for effectively loading ADM molecules on scFv molecules. (E) A nanodisk composed of reconstituted high-density lipoprotein particles and apo-AI–anti-CD20 scFv fusion peptide to specifically deliver curcumin to CD20-positive non-Hodgkins lymphoma (NHL) cells. The fusion peptide constitutes the protein scaffold of nanodisk and binds NHL cells through its scFv moiety. The structure of nanodisk has been derived from the work of Crosby and colleagues (71).

other VH-linker-VL domains on the same strands to form an active scFv. The resultant construct, called diabody, has antigen-binding sites twice that of a monovalent scFv (Figure 3A). Kiesgen and colleagues compared the capacities of scFv and diabody formats of an anti-EGFR antibody in delivering ranpirnase to EGFR-expressing cells and tumors (ranpirnase is an RNase inhibitor-resistant ribonuclease capable of degrading tRNA molecules) (42). They produced a scFv, a diabody and two fusion proteins, ranpirnase-scFv and ranpirnase-diabody, and evaluated their cytotoxicity for various EGFR-expressing cells. They found that enzymatic activity of ranpirnase-scFv was a little lower than that of ranpirnase alone. The enzymatic activity of ranpirnase-diabody was very considerable, nearly twice the activity

of ranpirnase-scFv. Both ranpirnase-diabody and ranpirnase-scFv entered the cell more rapidly than cetuximab. Ranpirnase induced a significant cytotoxicity in both EGFR-positive and EGFR-negative cells, indicating that it enters the cell in an EGFR-independent manner. The capacity of ranpirnase for cytotoxicity induction was not much less than that of ranpirnase-scFv, but it was strikingly lower than that of ranpirnase-diabody (42). The results all indicate that diabodies can serve as suitable carriers for targeted drug delivery.

Using scFv to Increase Tumor-Killing Activity of Granulocytes. TNF-related apoptosis-inducing ligand (TRAIL) is a cytokine protein involved in apoptosis induction in tumor cells (43,44). Studies have indicated that the truncated form of TRAIL (sTRAIL) produced by enzymatic cleavage of its extracellular domain still retains proapoptotic activity and can be employed to increase antitumor capacity of T cells and leukocytes (45,46). Phagocytes express a surface antigen, called C-type lectin-like molecule-1 (CLL1), which can be exploited for targeted drug delivery to these cells. Wiersma and colleagues produced a fusion peptide composed of an anti-CLL1-scFv and sTRAIL to specifically deliver TRAIL to granulocytes (47). They found that the fusion peptide, called CLL1:TRAIL, was able to specifically bind to and increase the antitumor activity of granulocytes. CLL1:TRAIL binds to granulocytes through its scFv moiety and triggers apoptosis through TRAIL. To examine whether CLL1:TRAIL synergizes the anticancer effect of monoclonal antibodies, they co-treated FaDu (EGFR-expressing cells) and Ramos B cells (CD20-expressing cells) with CLL1:TRAIL-armed granulocytes and a relevant monoclonal antibody (cetuximab and rituximab, respectively). In both cases, apoptosis was increased significantly, indicating a potential role of antibody-dependent cell-mediated cytotoxicity in synergizing the anticancer activity of CLL1:TRAIL (47).

Enhanced Delivery of Doxorubicin Using Liposomes Armed With a Bispecific scFv

Diabodies, which are composed of two different scFv blocks, can simultaneously target two different antigens on the cell surface. To determine whether such diabodies can improve drug delivery efficiency, Rabenhold and colleagues produced two scFv molecules (one targeting CD105 and the other targeting FAP) and one single-chain bispecific diabody (scDb, containing the two scFvs together; Figure 3B) and evaluated their capacity in delivering liposome-encapsulated doxorubicin to the target cells (48). They modified scFv molecules to contain a sulfhydryl group offering the potency to couple with another scFv. ILs derived from the scFv and diabody molecules were examined on different cell lines, including the cell line expressing CD105 (HUVEC cells) and a fragment antigen-binding (Fab)-transfected fibrosarcoma cell line (called HT1080-Fab) expressing both CD105 and FAP. The liposomes with anti-FAP alone, which they called scFv'FAP-IL, were unable to bind HUVEC cells, while those carrying anti-CD105 scFv [including monospecific anti-CD105 scFv ILs (called scFv'CD105-IL), bispecific ILs (combination of the two ILs, called scFv'FAP/CD105-IL) and bispecific single-chain diabodies (called scDb'CD105/FAP-IL)] were capable of binding these cells. To the T1080-Fab cells, the maximum interaction belonged to scDbCD105/FAP-IL and then to scFv'FAP/CD105-IL. In contrast, the minimum interaction belonged to scFv'CD105-IL and then to scFv'FAP-IL. Compared with unarmed liposomes (control liposomes), the CD105-carrying ILs all were able to induce considerable cytotoxicity in HUVEC cells (up to 59% reduction in cell viability), whereas scFv'FAP-IL could not affect cell viability significantly. For HT1080-Fab cells, scDbCD105/FAP-IL has been relatively more toxic than other ILs but less toxic than free doxorubicin (48). These results indicate that diabodies may be superior to single-chain antibodies for use as targeting ligands of drug carriers.

Targeted Delivery Using A Disease-Specific Cell Surface Receptor and scFv

Cell- or tissue-specific antigens provide an opportunity to specifically deliver anticancer drugs to that cell or tissue, without affecting normal cells. One such antigen is the fetal acetylcholine receptor (fAChR) that is expressed in rhabdomyosarcoma (RMS), the most common soft-tissue sarcoma in children. Brehm and colleagues took advantage of this receptor for delivery of microtubule-associated protein (MAP) to RMS (49). They produced a fusion peptide composed of a fully humanized anti-fAChR scFv antibody (called scFv35) and MAP and evaluated its specificity and cytotoxicity for fAChR-positive cell lines (FL-OH1 and RD). The scFv35–MAP fusion peptide selectively recognized the fAChR-positive cell lines while it was unable to bind fAChR-negative cell line U937 (human histiocytic lymphoma cell line). Compared with the positive control fusion peptide (scFv35-Pseudomonas exotoxin A, scFv35-ETA), the scFv35-MAP displayed an increased internalization rate, but a decreased cytotoxicity, in fAChR-positive cell lines. Cytotoxicity assay using another fusion peptide (scFv35-SNAP, a negative control peptide) revealed that cytotoxicity was due to effector proteins (MAP and ETA) and not scFv35 moiety (49). Although scFv35-MAP was proven to be relatively less toxic than scFv35-ETA, MAP-based drugs still maintain great interest since they are potentially less immunogenic than those of foreign origin (such as bacterial exotoxins).

Dual-Targeting Carrier for Delivery of Doctexal to Ovarian Cancer Cells

Huang and colleagues designed a dual targeting carrier and evaluated its capacity in delivering the anticancer drug doctexal (TXT) to ovarian cancer cells (50). The carrier was composed of

three main parts: a central core made from iron oxide (Fe_3O_4) nanoparticles—offering magnetism-targeting function, β-cyclodextrin (β-CD) units—providing holes for encapsulation of TXT and an antiendoglin scFv antibody for selectively targeting of ovarian cancer cells. Endoglin is a cell membrane protein that can be used to target ovarian cancer cells. Magnetic targeting assay displayed that the carrier could be directed by an external magnetic field. ELISA with endoglin-expressing ovarian cancer cells Scov3 indicated that the scFv antibody of the carrier (containing TXT) retained its binding capacity to endoglin. Anticancer activity of the carrier has been found to be due to the TXT molecules encapsulated in β-CD since the TXT-less carriers did not affect the viability of Scov3 cells. TXT-loaded carriers were more cytotoxic than raw TXT and exhibited a sustained release of TXT (until 94 h). TXT-loaded carrier has not been toxic for HUVECs (50). These results demonstrate that the dual-targeting carrier is an efficient drug carrier system to specifically deliver TXT to ovarian cancer cells.

Selective Fluorescence Imaging of FAP-Expressing Tumors Using an scFv-Based IL

Exploiting a tumor-specific antigen can help selectively target cancer cells in tumor tissues. One such type of antigens is FAP α, a transmembrane protein with enzymatic activity that is expressed by tumor-associated fibroblasts. Ruger and colleagues took advantage of this protein to selectively and efficiently detect cancer cells. They used a type of liposome (containing DY-676-COOH dye) capable of emitting fluorescence only upon cellular uptake and degradation. For selective delivery to FAP-expressing cells, they used an anti-FAP scFv antibody as targeting ligand. The antibody was first conjugated to $MalPEG_{2000}$-DSPE micelles and then inserted into the liposomes to form anti-FAP ILs. The resultant ILs could selectively recognize FAP-transfected human fibrosarcoma cells (HT1080-mFAP cells) but they failed to recognize FAP-negative cell lines. In contrast, the control liposomes (containing cysteine instead of anti-FAP scFv) weakly interacted with HT1080-mFAP cells; they were able to enter neither HT1080-mFAP nor HT1080 cells (wild-type cells). These results indicate that the uptake of anti-FAP-ILs depends on the interaction of FAP (on the cancer cell surface) and anti-FAP scFv (on the liposomes). *In vivo* analysis of tumor xenografts of HT1080-mFAP and HT1080 cells have shown that anti-FAP-ILs can specifically bind to and image FAP-expressing tumors. Control liposomes, on the other hand, have been shown to accumulate in stromal phagocytes, which can be exploited for drug/dye delivery to these cells (25).

Use of Anti-FAP scFv for Specifically Delivering Deferoxamine to FAP-Expressing Cells to Reduce Collagen Deposition

Excessive deposition of ECM components results in fibrosis. One of the major components of ECM is collagen, whose deposition has been well documented in development of fibrosis (51). Iron is necessary as a coenzyme for activity of prolyl-4-hydroxylase (P4H), an enzyme involved in collagen synthesis (52). Deferoxamine is an iron chelator that can inactivate P4H and reduce collagen deposition; however, its poor absorption and short half-life limits its therapeutic efficacy. To overcome these problems, Schuster and colleagues encapsulated deferoxamine molecules in PEG-functionalized ILs (scFv-conjugated liposomes) that were able to specifically target FAP-expressing cells. The scFv contained an additional cysteine residue within its linker region that allowed site-directed conjugation to liposomes (53). A cysteine residue within linker region serves as a suitable site for conjugation (Figure 3C). The influence of cysteine residue position on the success of conjugation process and activity of resultant ILs has been previously studied by Messerschmidt and colleagues (54). FAP is a serine protease selectively expressed in the stromal fibroblasts of epithelial cancers. Fibroblasts of normal adult tissues express undetectable levels of FAP (55). To generate an *in vitro* model of fibrosis, Schuster and colleagues isolated primary normal human lung fibroblasts from adult lung tissue and treated them with transforming growth factor β1 (TGF β1; to induce fibrosis-stimulating conditions), and ascorbate and proline (for collagen synthesis) (53). Unlike deferoxamine-loaded nontargeted liposomes, which were not able to decrease collagen deposition, deferoxamine-loaded ILs significantly reduced collagen deposition (Figure 3C). Free deferoxamine did not reduce collagen deposition (53). These results indicate that anti-FAP scFv plays a significant role in delivering deferoxamine molecules to fibroblasts.

USE OF SCFV MOLECULES FOR DELIVERY OF INORGANIC NANOPARTICLES

scFv-Mediated Targeted Delivery of As_2O_3-Nanoparticles to Liver Cells to Inhibit Tumor Angiogenesis

Vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis in different human cancers (56); therefore, it is regarded as suitable target for cancer therapy. Among chemical therapeutics, arsenic (As_2O_3) is widely used for cancer therapy owing to its potent anticancer activity (57–59); however, it lacks specificity to cancer cells and induces toxicity in normal cells. To specifically deliver As_2O_3 nanoparticles to liver cancer cells, Xiangbao and colleagues produced a humanized anti-VEGFR-2 scFv antibody (via ribosome display technique) and evaluated its ability to deliver arsenic to liver cancer xenografts (60). Humanized antibodies have lower immunogenicity than nonhuman antibodies (9) and reasonably are preferred for use as a drug carrier in the human body. As_2O_3-loaded scFv (anti-VEGFR-2-scFv-As_2O_3) significantly increased the concentration of As_2O_3 in tumor tissues of nude mice and

prolonged survival time. Survival time in mice treated with As_2O_3-loaded scFv was longer than that in mice treated with As_2O_3 nanoparticles or scFv alone (60). Anti-VEGFR-2 scFv-As_2O_3 has been found to significantly suppress the proliferation, invasion and migration of human hepatoma cancer cell line Bel 7402. The antitumor capacity of anti-VEGFR-2 scFv-As_2O_3 has been proven *in vivo* as well, where it was able to inhibit the growth of Bel 7402 tumor xenografts in BALB/c nude mice (61).

USE OF SCFV FOR DELIVERY OF IRON OXIDE NANOPARTICLES FOR TUMOR IMAGING

Selective delivery of imaging agents to tumor tissue makes it possible to selectively image that tumor. Kanazaki and colleagues produced a trastuzumab-based scFv and used it to selectively deliver iron oxide nanoparticles (IONPs) of different sizes (20, 50 and 100 nm) to HER2-expressing cells and tumor xenografts (62). IONPs have a biodegradable nature and represent low cytotoxicity, making them a favorable probe for photoacoustic cancer imaging. Among the scFv-conjugated IONPs (they named the resultant conjugations, based on the size of their IONPs, SNP20, SNP50 and SNP100), SNP20 displayed the highest binding affinity to, and the highest accumulation rate in tumor xenograft of, N87 cells (HER2-expressing human gastric cancer cells). In N87 tumor-bearing mice, SNP20 injection notably increased photoacoustic signal, but failed to increase the signal in mice bearing the xenograft of SUIT2 cells, pancreatic cancer cells expressing low levels of HER2. Furthermore, 20-nm IONPs alone (without scFv) increased photoacoustic signal in neither N87 nor SUIT2 tumors. Berlin Blue staining of N87 and SUIT2 tumors treated with SNP20 has revealed that N87 tumors uptake relatively greater amounts of IONPs (62). These results all indicate that scFv offers a high degree of specificity toward HER2-expressing cells/tumors and can serve as an efficient carrier of IONPs for imaging purposes.

USE OF SCFV TO INHIBIT THE RELEASE OF PAIN-RELATED PEPTIDE IN SENSORY NEURONS

Botulinum toxin type A (BTX-A) has gained enormous attention as a pain reliever since it can cleave synaptosomal-associated protein 25 (SNAP-25) and therefore prevents pain-related peptides from being released (63). Nociceptive sensory neurons express high levels of a surface receptor, called P2x purinoceptor (P2x3), which can be engaged to specifically target these cells. Ma and colleagues exploited this receptor for selectively delivering BTX-A to nociceptive sensory neurons (64). They designed and produced a fusion peptide composed of an anti-P2x3 scFv (called MH7C) and a part of BTX-A (including protease light chain, translocation and N-terminal half moiety of the binding domain) and evaluated its specificity for, and SNAP-25 cleaving activity in, dorsal root ganglion (DGR) neurons. The fusion peptide (called LC-H_N-H_{CN}/A-MH7C) specifically recognized DGR neurons and significantly increased the cleavage of SNAP-25. The LC-H_N-H_{CN}/A-MH7C-mediated cleavage occurred at strikingly lower concentration when compared with the control protein (LC-H_N-H_{CN}/A). The fusion peptide exhibited a high degree of safety for mice in such a way that it did not cause any mortality or behavioral disorders in mice (~25 g) even at high concentration (200 μg), whereas BTX-A caused mortality at significantly lower concentration (20 pg) (64). These results indicate that scFv-mediated drug delivery to sensory neurons not only decreases the drug dosage necessary for pain relief but also reduces the toxin-related mortality.

TARGETING LIGANDS OF DOXORUBICIN-LOADED $IONP_S$ DOES NOT CAUSE A IMMUNE RESPONSE

A potential drawback associated with the use of targeting ligands on the surface of drug carrier system is to induce ligand-specific antibody responses, which may disturb the cellular uptake of the carriers (65). Yang and colleagues applied an anti-EGFR scFv on the surface of IONPs to examine whether it could induce immune responses (66). To produce scFv-conjugated INOPs, they coated the surface of IONPs with an amphiphilic copolymer layer containing active carboxyl groups, which served as conjugation sites for amine PEG carboxyl to generate PEG-modified IONPs with surface carboxyl groups. These carboxyl groups, in turn, served as conjugation sites for scFv molecules to produce scFv-conjugated INOPs (66). Intravenous injection of near-infrared (NIR)-830 dye-labeled-scFv-conjugated IONPs to Balb/c mice bearing 4T1 mouse mammary tumors led to accumulation of the nanoparticles in tumor tissues. Repeated injection (two times, 200 pmol each) of scFv-conjugated IONPs resulted in high-level secretion of anti-scFv-specific antibody. No antibody secretion was detected in the serum samples of the mice injected with nontargeted nanoparticles. Interestingly, when Yang and colleagues encapsulated doxorubicin molecules in the scFv-conjugated IONPs and applied them on the mice, they found a significant decrease in murine anti-scFv-specific antibody secretion (66). These results indicate that the antibody response against scFv is not an issue for development of scFv-conjugated doxorubicin-loaded nanoparticles for cancer therapy (66).

USE OF SCFV AND DEXTRAN TO INCREASE DRUG LOADING EFFICIENCY

The number of loaded drug molecules per drug carrier unit can be an index of efficiency of that system for drug delivery. Chen and colleagues used a glucan compound, dextranT10, as a linker to load adriamycin (ADM) molecules on hepatocellular carcinoma-specific scFv molecules (scFv-SA3; Figure 3D) (67). They found that each scFv in the conjugate (ADM-Dextran–scFv-SA3) bound to ~14 ADM molecules. The conjugate significantly reduced the proliferation and colony formation of HepG2 cells *in vitro* and decreased the growth of tumor xenografts in nude mice. The conjugate was unable to bind the negative

Table 1. Summary of a number of studies concerning the use of single-chain antibodies in drug delivery systems.

Type of carrier/drug delivery system	Targeting ligand	Evaluated *in vitro/in vivo*	Result(s)	Ref.
Temozolomide (TMZ)-loaded IL (scL-TMZ)	anti-transferrin receptor scFv	U87, U251, U87R (a TMZ-resistant subclone of U87) Mice bearing glioblastoma multiforme tumors	Enhanced killing activity against U87 and U251 cells (compared with noncapsulated TMZ), sensitization of U87R to TMZ. Prolonged animal survival time	(72)
Fusion peptide composed of scFv format of PiPP mAb and pseudomonas exotoxin (PE38)	human chorionic gonadotropin (hCG)-specific scFv	hCG-expressing cells (U937, MOLT4 and A549 cells)	killing of hCG-expressing cancer cells without affecting PBMCs	(73)
Fusion peptide composed of viral interleukin-10 (vIL-10), MMP cleavable linker and anti-ROS-CII scFv	An scFv-targeting ROS-modified collagen type II (anti-ROS-CII scFv)	mouse MC-9 mast cells + arthritic C57BL/6 mice	Increased cell proliferation + Specific localization in arthritic knee + reduced inflammation	(74)
Mixing of doxorubicin–loaded mPEG-NPs and BsAbs (noncovalent binding)	humanized 15-2b anti-mPEG Fab fragment + human anti-EGFR scFv/anti-HER2-scFv	SW480 ($EGFR^+$ cells) and SK-BR-3 ($HER2^+$ cells BALB/c nude mice bearing xenograft of SW480/SW620 cells	Preferential binding to $EGFR^+$/$HER2^+$ cancer cells Significantly increased suppression of tumor growth when compared with nontargeted nanoparticles	(75)
Azido-functionalized protein nanocapsules loaded with GFP	Anti-HER2-scFv	SKBR3 (HER-2-overexpressing cell line) and MDA-MB-231 (triple-negative cell line)	Specific delivery of GFP to SKBR3 cells + no florescence in MDA-MB-231 cells	(76)
scFv-CD40L fusion peptide (EpCAM:CD40L)	Anti-EpCAM scFv	EpCAM-transfectant cell line HEK293. EpCAM	Induction of paracrine CD40 signaling in tumor-resident immature dendritic cells (iDC), maturation of DCs + activation of T cells.	(77)
scFv-CD40L fusion peptide (-CD20:CD40L)	Anti-CD20 scFv	$CD20^+$ leukemic B cells (BJAB and Raji)	Induction of CD40 signaling, induction of cell death	
Fusion peptide composed of Bluetongue virus VP2 protein and APCH	APCH (an scFv recognizing antigen-presenting cells)	guinea pigs, $IFNAR^{-/-}$ mice, and cattle	Enhanced humoral and cellular immune response in $IFNAR^{-/-}$ mice (when compared with VP2 alone + high titer of specific NAs in pigs and cattle	(78)
Fusion peptide composed of an anti-EGFR VHH and iRGD (a tumor-penetrating peptide, CRGDK GPDC)	VHH (variable domain of heavy chain of anti-EGFR antibody)	2D culture, multicellular spheroids (3D) culture, and tumor xenograft of BGC-823 cells (Human gastric adenocarcinoma cell line)	Enhanced antitumor activity when compared with anti-EGFR antibody alone + penetration into deeper zone of multicellular spheroids and tumors + …	(79)
crosslinked albumin nanoparticles targeting HGFR (c-Met)	Anti-HGFR nanobody (Anti-c-Met nanobody)	TOV-112D (Met-negative cells), TOV + Met (Met-transfected TOV-112D cells), MKN45, A549, A431	Specific binding to Met-expressing cells + internalization and lysosomal degradation of Met-targeted nanoparticles (evaluated in MKN45 and A549 cells)	(80)
Fusion peptide composed of scFv and nuclear localization signal (NLS)	scFv-targeting nuclear export signal (NES, C-terminus of mutated $NPMc^+$)	NPMc + -transfected HeLa cells and OCI-AML3 (human acute myeloid leukemia cells)	Accumulation of fusion peptide in the nucleus + effective binding to $NPMc^+$ in the cytoplasm + failing to relocate $NPMc^+$ in the nucleus	(81)
Rous sarcoma virus-like particles Displaying scFv antibody	scFv format of humanized CC49 antibody (anti-TAG-72 antibody)	LS174T human colon adenocarcinoma Cells (TAG-72-expressing cells) + HEK293 cells (control cells)	Selectively killing of LS174T cells by FITC/DOX-loaded carrier	(82)

Continued on next page

Table 1. ***Continued.***

scFv-TR1 fusion protein	scFv-specific for mesothelin, a tumor marker	wild-type Jurkat cells + J-Meso (Jurkat cells engineered to express human mesothelin) + OVCAR3 (ovarian cancer cell line-expressing mesothelin)	Selectively killing of mesothelin-positive cells	(83)

APCH, antigen presenting cell homing; BsAbs, bispecific antibodies; DOX, doxorubicin; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; H5N1, Highly Pathogenic Avian Influenza A; HGFR, hepatocyte growth factor receptor; MMP, matrix-metalloproteinase; mAb, monoclonal antibody; mPEG-NPs, methoxy PEGylated nanoparticles; NAs, neutralizing antibodies; PBMCs, peripheral blood mononucleated cells; ROS, reactive oxygen species; TAG-72, tumor-associated glycoprotein-72; 2D culture, two-dimensional monolayer culture.

control (QSG-7701 cells), indicating the role of scFv molecules in specific binding to HepG2 cells. The conjugate was more potent than either ADM alone or ADM/scFv-SA3 combination (67). Collectively, the results indicate that dextranT10 can be used as an efficient linker for drug loading on scFv molecules.

ADDITION OF SCFV TO PROTEIN SCAFFOLD OF LIPOPROTEINS TO OFFER SPECIFICITY TOWARD TARGET CELLS

Reconstituted high-density lipoproteins (rHDLs) have been widely used to deliver small drug molecules to cells. rHDL have a protein scaffold usually consisting of apolipoproteins (summarily called apo) (68–70). The major drawback correlated with rHDL-mediated drug delivery is the lack of specificity for the cells to which the drug is going to be delivered. Crosby and colleagues used an engineered protein scaffold to overcome the lack of specificity associated with rHDL-mediated drug delivery (Figure 3E) (71). They designed a fusion peptide composed of an anti-CD20 scFv and human apo A-I and evaluated its capacity for curcumin delivery to either CD20-positive or CD20-negative cells. They found that curcumin accumulated only in the CD20-positive cell lines (non-Hodgkin's lymphoma cells: Ramos and Granta cell lines). Fluorescence-activated cell sorting analysis revealed that the fusion peptide bound to CD20-positive cell lines through its anti-CD20 scFv section. The fusion peptide was unable to bind the CD20-negative cell line (Jurkat) substantially (71). These results all indicate that the scFv moiety is responsible for specificity of the fusion peptide for CD20-positive cells, guaranteeing a targeted drug delivery to these cells.

In addition to the carriers described above, a number of fusion peptides and other types of carriers (including liposome-based and non–liposome-based carriers) have been designed and evaluated for targeted drug delivery; however, because of space limitations, we do not describe them in detail. A summary of these studies is presented in Table 1.

CONCLUSION

scFvs can serve as targeting ligands on the surface of encapsulated drugs or in infusion with a protein drug for specific drug delivery. They can also be used for effectively loading a drug into the cells of interest, as we saw in the case of adriamycin delivery to hepatocellular carcinoma cells. Monospecific scFv diabodies can increase the specificity, internalization rate and cytotoxicity of drugs, offering an enhanced possibility to treat cancer. Bispecific scFv diabodies, which simultaneously bind to two different antigens on a cell surface, can help increase the efficiency of drug liposome-based delivery systems and therefore improve the therapy of diseases. scFv molecules in fusion with cell-penetrating peptides (e.g., truncated protamine and arginine-rich peptides) or some viral peptide (e.g., transmembrane domain of F-protein of Sendai virus) are potentially able to enhance the cell penetration rate and hence the accumulation of drug within the target cells. Collectively, the results of the studies included in this review indicate that scFv molecules have a high potential for use as targeting ligands, either on the surface of encapsulated drugs or in fusion with therapeutic proteins. scFv molecules can be conjugated to carriers by either covalent or noncovalent bonds. Cysteine residues within linker region or after His-tag (outside of scFv molecule) can serve as a suitable site for site-directed conjugation, allowing the scFv to make a strong linkage with carriers without losing its affinity. The results presented in this review indicate that scFv molecules are efficient targeting ligands for use in drug delivery systems. Some scFv-conjugated drug delivery systems discussed in this review have been studied only *in vitro*, while others have been further evaluated in animal models (tumor xenografts). Many drugs are efficacious in *in vitro* conditions, but few of them display efficacy in tumor xenografts (animal model) and fewer in clinical trials; therefore, the scFv-conjugated drug delivery systems presented in this review need to be further evaluated in animal model and/or clinical trials to be approved for therapeutic applications.

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DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular*

Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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